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Human adipose-derived mesenchymal stem cell spheroids improve recovery in a mouse model of elastase-induced emphysema

Ryeon Jin Cho[1,#], *You-Sun Kim*[1,2,#], *Ji-Young Kim*[2] *& Yeon-Mok Oh*[1,2,3,*]

[1]University of Ulsan College of Medicine, Seoul 05505, [2]Asan Institute for Life Science, Seoul 05505, [3]Department of Pulmonary and Critical Care Medicine, and Clinical Research Center for Chronic Obstructive Airway Disease, Asan Medical Center, Seoul 05505, Korea

Emphysema, a pathologic component of the chronic obstructive pulmonary disease, causes irreversible destruction of lung. Many researchers have reported that mesenchymal stem cells can regenerate lung tissue after emphysema. We evaluated if spheroid human adipose-derived mesenchymal stem cells (ASCs) showed greater regenerative effects than dissociated ASCs in mice with elastase-induced emphysema. ASCs were administered via an intrapleural route. Mice injected with spheroid ASCs showed improved regeneration of lung tissues, increased expression of growth factors such as fibroblast growth factor-2 (FGF2) and hepatocyte growth factor (HGF), and a reduction in proteases with an induction of protease inhibitors when compared with mice injected with dissociated ASCs. Our findings indicate that spheroid ASCs show better regeneration of lung tissues than dissociated ACSs in mice with elastase-induced emphysema. [BMB Reports 2017; 50(2): 79-84]

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the fourth-leading cause of death worldwide and includes cases of chronic bronchitis and emphysema. Emphysema is characterized by alveoli damaged by external factors, such as smoking (1). Matrix metalloproteases (MMPs) are extracellular matrix (ECM)-associated enzymes involved in the synthesis and degradation of connective tissue components for tissue remodeling and repair (2). A recent study determined that secreted MMPs (MMP-2, 9, and 12) contributed to the development of emphysema by degrading the alveolar wall matrix (3). In emphysema, regeneration of the alveolar wall is impaired; however, a mesenchymal stem cell (MSC) therapy for this condition is currently under development. MSCs that have been isolated from various organs, such as bone marrow, adipose tissue, and umbilical cord blood, can induce tissue repair via self-renewal, differentiation, and paracrine effects (4). The recovery effects of MSCs have been confirmed in various lung disease models, including lipopolysaccharide (LPS)-, cigarette smoke-, and bleomycin-induced lung injury models (5-7).

We previously tracked adipose-derived mesenchymal stem cells (ASCs) that were injected *in vivo* via intravenous routes. Labeled ASCs were detected for up to 24 h post-injection (8). Moreover, only 17% of cardiac sphere-derived stem cells survived up to 1 h after injection, and therapies using dissociated stem cells showed complications, including cell loss, in a myocardial infarction model (9, 10).

A spheroid is a self-assembled group of cells, and many studies have emphasized the differences between 2D and 3D culture environments, such as nutrient and oxygen gradients, cell-to-cell interactions, and matrix deposition (11, 12). A recent study showed that spheroids increase anti-apoptotic and anti-inflammatory properties both *in vitro* and *in vivo* (13). In this report, we used a mouse model of elastase-induced emphysema. Mice received intrapleural injections of aggregated "spheroid" ASCs to compare their therapeutic efficacy with that of dissociated ASCs.

RESULTS

Characterization of dissociated and spheroid ASCs

A monolayer of ASCs cultured for 3 days (Supplementary Fig. 1A) were seeded into PDMS- concave microwells coated with 3% BSA. Five minutes after seeding, unattached ASCs were removed using the ASC-conditioned medium. Cells began to aggregate within 1 h of seeding (Supplementary Fig. 1B). Generally speaking, homogeneous spheroid ASCs were observed after 24 h (Supplementary Fig. 1C).

*Corresponding author. Tel: +82-2-3010-3136; Fax: +82-2-3010-4650; E-mail: ymoh55@amc.seoul.kr
#These authors contributed equally to this work.

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To characterize the dissociated and spheroid ASCs, we measured the expression of cell survival-related apoptosis proteins and growth factors in protein samples extracted from ASCs. Bcl-2 inhibits interactions between mitochondria and BAX in the cytosol. The Western blot images suggested that BAX expression was similar in spheroid and dissociated ASCs, whereas Bcl-2 expression was increased in spheroid ASCs (Fig. 1A). ImageJ was used to quantify protein levels. We determined that BAX expression was significantly decreased and BCL2 expression was significantly increased in spheroid ASCs when compared with dissociated ASCs. Furthermore, the Bcl-2/BAX ratio was significantly increased in spheroid ASCs (Fig. 1B). FGF-2 and VEGF levels were also significantly increased in spheroid ASCs (Fig. 1C and D). To measure the levels of secreted growth factors, the culture medium of dissociated and spheroid ASCs was concentrated, and growth factors were measured by ELISA (Fig. 1F). VEGF secretion was increased in spheroid ASCs when compared with dissociated ASCs. FGF2 was not detected in the media from either group.

Spheroid ASCs improved lung recovery in an elastase-induced mouse model of emphysema

Emphysema was induced by elastase in C57BL/6 mice (day 0). After 7 days, mice were intrapleurally injected with 1×10^5 dissociated or spheroid ASCs. On day 14, mice were euthanized (Fig. 2A), and lung tissues were collected. The H and E-stained lungs of the elastase-treated group showed severe alveolar destruction (Fig. 2C) when compared with the control group (Fig. 2B). The mice treated with the two types of ASCs showed recovery from the alveolar damage (Fig. 2 D and E). We used the mean linear intercept (MLI) method to quantify this recovery. The MLI was reduced in the dissociated ASC-infused group (97.9 μm) and spheroid ASC-infused group (78.2 μm) when compared with the elastase only group (109.6 μm). The

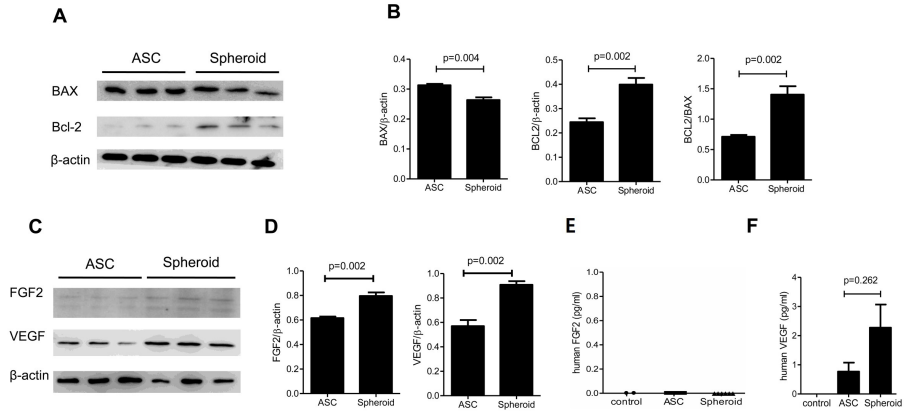

Fig. 1. The expression of apoptotic markers and growth factors. (A) Western blotting images and (B) protein band quantification for BAX and Bcl-2. (C) Western blotting images and (D) protein band quantification for FGF-2 and VEGF. (E) FGF-2 and (F) VEGF production was measured in conditioned media.

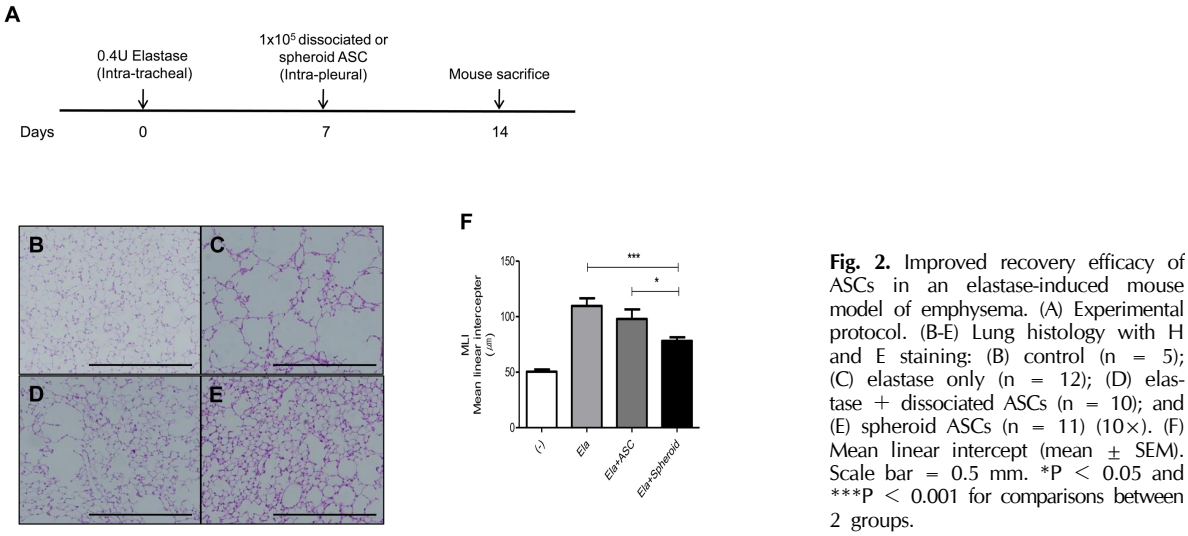

Fig. 2. Improved recovery efficacy of ASCs in an elastase-induced mouse model of emphysema. (A) Experimental protocol. (B-E) Lung histology with H and E staining: (B) control (n = 5); (C) elastase only (n = 12); (D) elastase + dissociated ASCs (n = 10); and (E) spheroid ASCs (n = 11) (10×). (F) Mean linear intercept (mean ± SEM). Scale bar = 0.5 mm. *P < 0.05 and ***P < 0.001 for comparisons between 2 groups.

MLI of the spheroid ASC-infused group showed a stronger reduction than the dissociated ASC-infused group. These findings suggest that the recovery between the dissociated and spheroid ASC groups differed (Fig. 2F).

Effects of injected ASCs on the lungs of mice with elastase-induced emphysema

The harvested lung tissues from mice with or without elastase-induced emphysema were analyzed by ELISA and Western blotting (data not shown) to identify changes in host cells after the transplantation of dissociated or spheroid ASCs. The protein levels of mouse FGF-2, HGF, and VEGF were quantified using ELISA. Mouse FGF-2 was significantly increased in the spheroid ASC-injected group when compared with the dissociated ASC-injected group (Fig. 3A). Mouse HGF and VEGF levels were not significantly different between dissociated and spheroid ASC-injected groups (Fig. 3B and C). The growth factor levels in the three treatment groups were not significantly different from the control (-) group.

Effects of injected ASCs on the regulation of MMP production

To observe the effects of ASCs on tissue regeneration, the mRNA transcript levels of MMP-2, -9, and -12 in lung tissue were measured by qPCR. MMP-2 mRNA expression tended to be lower in the injected ASC group when compared with the negative control group. MMP-12 expression was significantly lower in the spheroid ASC-injected group when compared with the dissociated ASC-injected group (Fig. 4A). Protein expression levels were evaluated by Western blotting (Fig. 4B) and zymography (Fig. 4C). MMP-9 expression at the mRNA and protein level was similar between the dissociated and spheroid ASC-injected groups. Both groups also showed reduced MMP-2 expression and activity. Additionally, the spheroid ASC-injected group showed a greater reduction in MMP-2 activity when compared with the dissociated ASC injected group. To extend these findings, we tested the expression levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) and secretory leukocyte protease inhibitor (SLPI), which both inhibit MMPs (Fig. 4D). Surprisingly, the injection of either dissociated or spheroid ASCs significantly increased the mRNA transcript levels of TIMP-1, which encodes a protease inhibitor. The transplantation of spheroid ASCs resulted in a greater increase in TIMP-1 and SLPI expression when compared with dissociated ASCs.

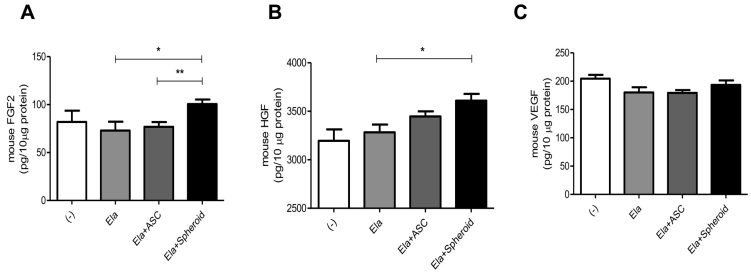

Fig. 3. Growth factor production in lungs with emphysema: (A) FGF-2, (B) HGF, and (C) VEGF. *P < 0.05 and **P < 0.01 for comparisons between 2 groups.

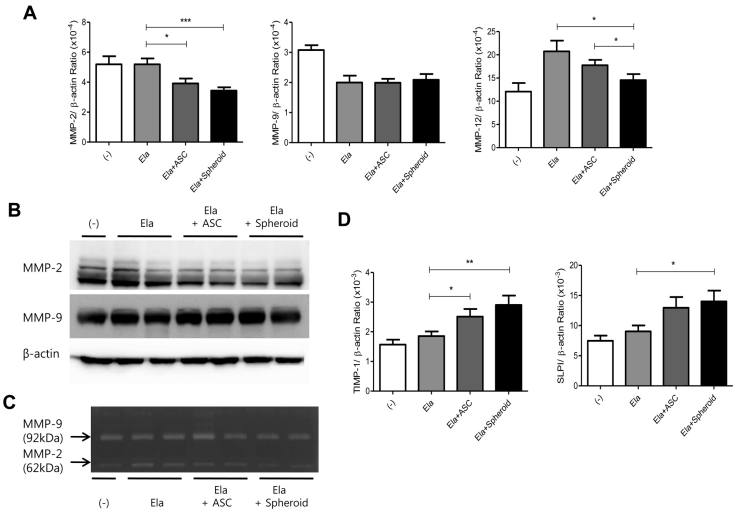

Fig. 4. Effects of ASCs on MMPs, TIMP-1 and SLPI expression in lungs with emphysema. (A) The mRNA levels of MMP-2, MMP-9, and MMP-12 in the lung. (B) and (C) Representative images for western blotting and gelatin zymography. (D) The mRNA expression of TIMP-1 and SLPI. *P < 0.05, **P < 0.01, and ***P < 0.001 for comparisons between 2 groups.

DISCUSSION

In the current study, we examined if spheroid ASCs showed improved therapeutic efficacy in a mouse elastase-induced model of emphysema. Classically, MSCs have been cultured as a two-dimensional (2D) monolayer using coated culture plates. However, 2D cultures are disadvantaged by the fact that MSCs lose their stemness properties (e.g., replication potential and differentiation capacity) when grown in this manner; notably, this phenomenon does not occur in humans or mice *in vivo* (14). To overcome this problem, many researchers have tried to adopt three-dimensional (3D) spheroid cultures. The effect of spheroid formation on cellular differentiation and cell-cell interactions has been studied. The formation of a spheroid prevented apoptosis, and thus facilitated differentiation, because of the conservation of cell-cell interactions that were important for survival and colony formation (15, 16).

Here we compared the expression of apoptotic and growth factors between dissociated and spheroid ASCs. A previous report showed that spheroid ASCs decreased BAX protein expression when compared with dissociated ASCs (17). Our Western blot results showed no significant difference in BAX protein levels and increased Bcl-2 protein expression in spheroid ASCs when compared with dissociated ASCs. The correlation of BAX and Bcl-2 expression was not determined.

Growth factors perform an important role in tissue regeneration. FGF-2 is a basic fibroblast growth factor involved in multiple biological activities, including angiogenesis, migration, proliferation, and anti-apoptosis (18-20). VEGF is involved in vascular regulation in angiogenesis, cell-cell and cell-matrix interactions, and the proliferation of endothelial cells in injured lung tissue. VEGF was reported as a crucial regulatory factor in the aggravation or recovery of emphysema (21-23). We observed an increase in FGF-2 and VEGF in cells and tissues without a release of FGF2 in the culture medium.

In various animal models, the major advantages of spheroid MSCs are their higher level of stemness and anti-inflammatory properties and exertion of stronger therapeutic effects, such as increased growth factor secretion in the recipient and greater differentiation capacity (13, 16, 17, 24). We did not assess stemness in our current study; however, the effects of injected spheroid ASCs were confirmed by the increased growth factor expression in the host tissue when compared with dissociated ASCs.

The use of spheroid ASCs in our current study significantly attenuated emphysema in a mouse elastase-induced model when compared with dissociated ASCs at a similar dose of 1×10^5 cells. To characterize this therapeutic mechanism, we measured growth factor and MMP expression in mouse lung tissue lysates.

The expression of each growth factor was significantly enhanced, and the expression of MMPs (-2 and -12) was diminished when compared with the dissociated ASC-injected group. In contrast, the expression and activity of the protease inhibitors TIMP-1 and SLPI have significantly enhanced in spheroid ASC-injected mice. Previous studies revealed that exogenous growth factors, i.e., EGF, VEGF, and HGF as an angiogenic factor, enhanced the expression of MMPs *in vitro* and *in vivo* via cell migration and proliferation and ECM remodeling (25-27). The MMPs (-2, -9, and -12) assayed in our current analyses are proteases with major roles in emphysema. These MMPs are secreted by various cells, including epithelial cells, neutrophils, eosinophils, and alveolar macrophages, and they can degrade matrix components in normal and abnormal states (28). MMP-12 helps macrophages to invade tissues and thus contributes to the degradation of the alveolar wall. In a recent report, the pathogenesis of emphysema was blocked at an early stage by ablating the MMP-12 gene or inhibiting MMP-9 and MMP-12 in a smoke-induced emphysema animal model (29-31). TIMP-1 inhibits the activity of most MMPs, including MMP-12, but not the membrane-type MMPs (-14, -15, -16, -17), whereas SLPI inhibits the activity of elastase (32). Some reports have shown that proteins secreted by MSCs can protect tissues by inhibiting the activity of endogenous and exogenous MMPs and proteases via the ERK1/2 pathway and other proteins, such as erythropoietin, TIPMs, and VEGF *in vitro* (2, 33, 34).

Our current findings suggest that dissociated or spheroid ASCs injected in a mouse elastase-induced model of emphysema inhibit MMPs via TIMP-1 and SLPI and induce regeneration via growth factor production. Moreover, the use of spheroid cells resulted in an improved therapeutic efficacy *in vivo* when compared with dissociated cells.

Spheroid ASCs enhanced recovery in an elastase-induced mouse model of emphysema by increasing growth factor production and anti-proteases when compared with dissociated ASCs at the same dose. Thus, spheroid ASCs have potential as a future treatment of emphysema.

MATERIALS AND METHODS

Mice

Pathogen-free female C57BL/6 mice were used at 6 weeks of age and with each one having a body weight of 20 g (Seongnam, Korea). All animals were cared for according to the guidelines of the Institutional Animal Care and Use Committee of Asan Medical Center (Seoul, Korea).

ASC culture

Human ASCs were purchased from Invitrogen (Carlsbad, CA, USA). ASCs were cultured in MesenPRO RS Medium supplemented with Growth Supplement (Invitrogen, CA, USA) and 1% penicillin at 37°C in a 5% CO_2 incubator. ASCs at passage 5 were used for all experiments.

Spheroid formation

Spheroids were formed using polydimethylsiloxane (PDMS)-based concave microwell molds. Concave microwells with a

diameter of 300 μm were coated with 70% (v/v) ethanol, followed by PBS and 3% (W/V) BSA to prevent cell attachment. Each concave microwell contained 64 holes and suspended ASCs were seeded at a density of 1×10^5 per well. After several minutes, the remaining cells were removed using ASC culture media with pipette. Concave micro-wells seeded with ASCs were incubated at 37°C in a 5% CO_2 incubator for 24 h.

An elastase-induced mouse model of emphysema and ASC transplantation

C57BL/6 mice were anesthetized intraperitoneally (i.p.) with 16 μl of Zoletil 50 (Virbac Laboratories, Carros, France) and 4 μl of Rompun (Bayer Korea, Ansan, Korea). Next, mice were intratracheally (i.t.) administered porcine pancreatic elastase (0.4 U, 8 U/ml; Sigma–Aldrich, St. Louis, MO, USA) at day 0. After 7 days, mice were intrapleurally injected with dissociated or spheroid ASCs (1×10^5 cells) using a syringe equipped with a 26-gauge needle.

Gene expression analysis by quantitative polymerase chain reaction (qPCR)

Total RNAs from lung tissues and ASCs were extracted using an RNeasy Mini Kit (Qiagen, Düsseldorf, Germany) and synthesized into cDNA using a cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). qPCR analyses were performed with a real-time LightCycler 480 with SYBR Green I master mix (Roche Diagnostics, CA, USA) and primers. The list of primers used is shown in Supplementary Table 1.

Detection of MMP activity assays using zymography

To detect MMP activity in the mouse lung, tissues were homogenized in a radioimmunoprecipitation assay buffer (RIPA; Cell Signaling Technology, Danvers, MA, USA) without a protease inhibitor and quantified using a BCA kit (Thermo Scientific, CA, USA). Samples containing 10 μg of protein were subjected to 10% SDS-PAGE using a gelatin-based gel (Sigma–Aldrich, MO, USA). To develop the zymogram, the gels were incubated in 1× zymogram development buffer (Bio-Rad, CA, USA) at 37°C for 12 h. Next, gels were stained with 0.5% Coomassie blue R-250 (Bio-Rad, CA, USA) in 30% ethanol and 10% acetic acid for 1 h. Destaining was performed with 30% ethanol and 10% acetic acid.

Enzyme-linked immunosorbent assay (ELISA)

A total of 10 μg of protein from mouse tissue and 30 μg of total protein from 1×10^6 ASCs were used for ELISAs (R&D Systems; Minneapolis, MN) according to the manufacturer's instructions. The measured proteins included human and mouse growth factors (FGF-2, HGF, and VEGF).

Western blotting

Lysates of tissues and cells were homogenized in RIPA buffer and quantified using the BCA assay. Thirty microgram samples were separated by SDS-PAGE using a 10-15% gel and then transferred to a PVDF membrane. Primary antibodies against BAX, Bcl-2, FGF2, VEGF, MMP-2, and MMP-9 (Cell Signaling Technology, Danvers, MA) and their corresponding secondary antibodies were incubated with the membrane overnight at 4°C and for 1 h at room temperature, respectively. Membranes were scanned with LAS 4000 (Fujifilm; Tokyo, Japan) using film to detect the protein signals.

Histology and quantification of emphysema

The histology of the lungs was completed using a previously described method (8). Briefly, the perfused lungs were inflated with 0.5% low-melting agarose, fixed with 4% formalin, and embedded in paraffin. Lung sections with a thickness of 6 μm were stained with H and E. The mean linear intercepts (MLI) were determined from the microscopic images.

Data analysis

Statistical analyses were performed using the GraphPad Prism ver. 5 (GraphPad software, La Jolla, CA, USA). The data are presented as the mean ± SEM. The Mann–Whitney test was used to compare groups, and statistical significance was set at $P < 0.05$.

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CONFLICTS OF INTEREST

Authors have no conflicting financial interests.

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